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Clostridium Botulinum Type E in Gulf Coast Shrimp and Shucked Oysters, and Toxin Production as Affected by Irradiation Dosage, Temperature, Storage Time and Mixed Spore Concentrations.

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CLOSTRIDIUM BOTULINUM TYPE E IN GULF COAST SHRIMP AND SHUCKED OYSTERS, AND TOXIN PRODUCTION AS AFFECTED BY IRRADIATION DOSAGE, TEMPERATURE, STORAGE TIME AND MIXED SPORE CONCENTRATIONS

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ABSTRACT

Of immediate concern to the U. S. Food and Drug Administration, U.S.P.H.S., is the potential danger of the presence of Clostridium botulinum, type E toxin in food. Amongst the many possible pathogens, C. botulinum ranks at the top as a serious and potent health hazard. It is unfortunate that many foods are susceptable to its growth and subsequent toxin production as the environment provides excellent physiological conditions at certain times during processing. Thus the presence of C. botulinum, type E toxin is a problem of national concern to all sections of the economy involved in food handling and processing and their presence or absence in Gulf Coast Shrimp and Oysters must be ascertained for the protection and safety of the consumer.

This investigation was initiated to isolate and identify C. botulinum type E toxin from Gulf Coast shrimp and oysters obtained from commercial processors and retail outlets. Furthermore, a study was conducted of the capability of irradiated and nonirradiated fresh headless Gulf shrimp and shucked oysters to support the growth of C. botulinum and type E toxin production, as affected by various low doses of gamma irradiation, temperature, storage time and the size of a mixed spore concentration.

The results of this research emphasized the importance of holding shellfish on ice at a temperature at 35°F rather than at

refrigerated temperatures around 40°F in order to assure maximum consumer safety.

The examination of more than 700 lbs of fresh Gulf Coast shrimp and more than 300 lbs of fresh shucked oysters for the presence of C. botulinum type E has indicated its absence.

Toxicity studies showed that no toxin was produced when fresh Gulf Coast headless shrimp and shucked oysters were stored on ice (33°F) up to 30 days, after being inoculated with mixed C. botulinum type E spores at 10^3 and 10^4 spores/g either without irradiation or with exposure to Co^{60} radiation at 150, 200, 300 and 500 Krads.

Fresh Gulf Coast headless shrimp were inoculated with mixed C. botulinum type E spores at 10^3 and 10^4 spores/g. Samples were subjected to Co^{60} radiation at 150, 200, 300, and 500 Krads. The irradiated samples along with nonirradiated controls were then stored at 42°F. After 7 and 14 days, toxin was found in all samples except those which had received 500 Krads; in these, toxin was demonstrated after 30 and 31 days.

Toxin production again was shown after 30 days when fresh shucked oysters were stored at 42°F after being inoculated with mixed C. botulinum type E spores/g and subjected to Co^{60} radiation at 150, 200, 300 and 500 Krads along with a nonirradiated control.

INTRODUCTION

Botulism has been defined by Dolman (11) as a relatively rare, tragic, and in some respects still mysterious, neuroparalytic disease affecting man and animals in various parts of the world. It generally results from the ingestion of a foodstuff containing toxic metabolites from one of several known types of anaerobic bacilli, Clostridium botulinum, whose natural habitat is the soil of certain regions.

The 1963 outbreak of botulism caused by C. botulinum in commercially canned tuna and vacuum packed smoked whitefish chubs resulted in a renewed interest in C. botulinum type E in the United States.

This investigation was undertaken to study the incidence of C. botulinum, type E toxin in samples of fresh, frozen, peeled and deveined and breaded shrimp and fresh, frozen, breaded and canned shucked oyster samples of the Gulf Coast States from commercial processors and retail outlets. The investigation also included a study of the capability of irradiated and nonirradiated fresh Gulf shrimp and shucked oysters to support the growth of C. botulinum and type E toxin production, as affected by various low doses of gamma irradiation, temperature, storage time and the size of a mixed spore concentration.

SURVEY OF THE LITERATURE

Geiger (19) stated that the first recognized outbreak of botulism was observed over 200 years ago, although Ermengen (17) reported that the causal organism was not isolated until 1859.

According to Meyer (32) human botulism has been found chiefly in North America, Europe and Japan, although two outbreaks have been recorded in Argentina and two in Australia.

The first outbreak of type E botulism caused by a native U. S. food was recorded in Minneapolis in 1960 (2). This outbreak resulted from the consumption of smoked ciscoes from Lake Superior.

Type E botulism has occurred in many parts of the world. Table 1 in the Appendix lists the known outbreaks through 1963 as reported by Dolman and Iida (13), Nakamura (38), and the Morbidity and Mortality Weekly Report (37).

C. botulinum type E, one of the causative agents of botulism outbreaks, has a world wide distribution. Kamizawa (26) and Kakamura et al. (25) have demonstrated its presence repeatedly in soil and mud samples on Hokkaido. Johannsen (20, 21) has isolated the organism from large numbers of soil, seashore, and sea bottom samples in and near Sweden. Pederson (43) has found it in soil and bottom mud in Denmark. Dolman (13) has isolated the organism repeatedly from bottom samples off the coast of British Columbia.

A review of Public Health Records of the Gulf States did not

reveal any cases of type E botulism attributed directly to Gulf States shrimp and oysters. Previous preliminary work on shellfish by Novak (40) indicated the absence of C. botulinum, type E toxin in shrimp and oysters.

Dolman (10) and Johannsen (22) reported that all known outbreaks of type E botulism have occurred in the Northern hemisphere, and with few exceptions have been associated with the consumption of fish or other animal products from the sea. Early theories that C. botulinum, type E toxin was limited to the marine environment have been refuted by reports of the occurrence in soils and fresh waters of France, (Prévot et al., 44), Japan, (Kanzawa, 26), Sweden, (Johannsen, 21), and British Columbia, (Dolman, 10).

Bott et al. (5) reported that the intestinal contents of more than 3,000 fish from Lake Erie, Superior, Huron, and Michigan were examined for C. botulinum type E. Demonstration of the organism was accomplished by identifying its toxin in liquid cultures inoculated with material from the alimentary tract. Incidence figures, expressed as per cent of the fish tested, were: Lake Erie, 1%; Lake Superior, 1%; Lake Huron, 4%; the main body of Lake Michigan, 9%; and Green Bay (on Lake Michigan), 57%. Thus, C. botulinum type E appeared to be widely but unevenly distributed in the Great Lakes, and fish from all these areas were potential carriers.

Johnston et al. (23) reported that pure culture isolates of C. botulinum type E were rapidly obtained from 40 of 41 individual packages of smoked whitefish chubs which were subjected to alcohol treatment.

Dolman (14) and Meyer (32) stated that there are now recognized

six distinct types of C. botulinum, as types A, B, C, D, E, and F which can be differentiated by the serological specificity of their toxins. Type C consists of two subtypes C_{α} and C_{β} which differ in their effects on various animal species and in several other features.

Dolman (14) also stated that types C and D only rarely have been implicated in outbreaks of human botulism, but they have caused huge losses in wild and domestic animals. Dolman (14) further stated that type F was first recognized in 1958 and has been involved in only one known outbreak from a home-prepared liver paste in Denmark. Eklund and Poysky (16) stated that C. botulinum type F has been demonstrated in two samples of marine sediments. One sample was taken at a depth of 83 meters off the coast of California; the other, 100 meters off the coast of Oregon. Cultures of this type have not been reported previously in the United States, and only one before in the whole world. Lamanna (29) and Sakaguchi et al. (46) reported that types A, B and E have been responsible for all but a few of the known outbreaks among humans.

In addition to their serological differences, types A and B are distinguishable from type E by several other characteristics, namely:

1. Heat resistance. Crisley (7), Dack (8) and Schmidt (51) reported that spores of type A and B survived boiling for several hours, whereas type E spores usually were killed by heating to 80°C for 30 minutes or less.

2. Minimum growth temperature. Michener and Elliott (35) showed that types A and B grow slowly if at all at 50°F, whereas certain strains of type E have been observed to grow as low as 38°F.

3. Toxicity of cultures and activation of toxin by trypsin.

Sakaguchi et al. (46) demonstrated that cultures of type E possessed much lower toxicities than type A and B when injected into mice. Foster et al. (16) stated that the potency of type E cultures could be increased 10 to 100 fold by treating with trypsin. These workers stated further that trypsin did not ordinarily "activate" the toxins of type A and B, presumable because these organisms produce their own proteolytic enzymes, which may have performed the same function as trypsin. However, Bonventre and Kempe (4) have shown that young cells of type A and B contained a toxic "precursor" that could be released by sonic disruption of the cells. The potency of this material, like that of type E toxin, could be increased by treatment with trypsin.

Riemann (45) showed that type E toxin was stable in acid but was readily destroyed by alkali and was inactivated at 80°C for 30 minutes.

Meyer (31) defined botulism as a specific intoxication with the toxins of C. botulinum or parabotulism. The six serotypes, A, B, C, D, E, and F exist in nature as sporulating saprophytes and grow freely in a great variety of inadequately preserved animal or plant foods. During resultant spoilage a powerful toxin is formed, which on ingestion, is absorbed, ultimately inducing some changes in the motor-nerve terminals at the neuromuscular junction; acetylcholine output was diminished, with effects resembling denervation.

The histories and data collected by Meyer et al. (34) showed that 500 single or group intoxications, in the United States from 1899 to December 1952, involved a total of 1,324 persons, of whom 846 died. The mortality rate, which has at times reached 67%, has

dropped only to 62%.

Table 2 in the Appendix shows that from 1899 through 1963 there were a total of 1,561 reported cases of botulism. The largest number of cases was reported in the decade 1930 through 1939; the second in the decade 1920 through 1929, and the third in the decade from 1940 through 1949. C. botulinum, type A and B toxin, were found most often to be responsible for the botulism outbreaks carried by non-commercially processed foods.

Up to 1925, commercially canned foods manufactured in the United States had been at fault in 32 cases, but intensive research, supported by the protective measures originally devised by the State of California, led to the adoption of scientific procedures by the canning industry.

Records relating to botulism due to consumption of commercially prepared foods were more accurate than were those relating to other than commercially prepared foods and are summarized in Table 3. Fifty-one outbreaks have been reported since 1906, with 109 deaths in a total of 219 cases.

A review of the foods involved in outbreaks since 1950 presented in Table 3 in the Appendix indicated that only 5 cases out of 44 were attributable to canned food products. The remaining 39 cases were associated with the consumption of products which received no heat treatment, such as cheese or smoked fish processed at temperatures incapable of destroying C. botulinum.

Type E C. botulinum has been the major cause of cases of botulism from commercially prepared foods in the past two decades as shown in Table 3 in the Appendix. A review of the known outbreaks of type E

botulism presented in Table 4 in the Appendix showed that the foods most often involved were those which were unprocessed, or processed at relatively low temperatures, or simply air dried.

Dolman and Iida (13) indicated in their report on type E botulism that the U. S. outbreaks fell into the following three main categories:

1. The implicated foodstuffs had been commercially processed and appeared normal. Four of five reported outbreaks were due to imported foods and one was attributed to eating vacuum-packed smoked fish caught in Lake Superior.

2. Salmon eggs served as the vehicle in six outbreaks among Pacific Coast Indians, with 11 persons affected and 6 deaths. These eggs were often prepared by simple fermentation for several days or weeks.

3. Six outbreaks occurred among Eskimos of the northwestern coast of Alaska. Twenty people were ill and 6 of them died. The implicated food in five outbreaks was muktuk-pieces of dried beluga (white whale) flipper, cured by several weeks of immersion in seal oil. The sixth outbreak was due to consumption of the fluke of a decomposing grey whale.

The 1963 outbreaks of botulism which were reported in the Morbidity and Mortality Weekly Report (37) are found in Table 5 in the Appendix. Twelve outbreaks totaling 46 cases, including 14 deaths, were reported. These 46 cases in 1963 represented the highest total for any one year since 1939 and the eighth highest since 1899. Commercially canned food products accounted for 19 cases with 7 deaths. Home canned foods resulted in 22 cases with 5 deaths.

Nickerson and Goldblith (39) reported that there had been no outgrowth and toxin production in any sample of shucked clams inoculated with C. botulinum type E, at 10^2, 10^4 and 10^6 spores/g of Alaska, Beluga, Minneapolis and E8 strains after irradiation at 350, 600 and 800 Krads or in unirradiated clams stored at $35^{\circ}F$ or lower. They further showed that shucked clams inoculated with 10^2, 10^4 and 10^6 mixed spores/g of the same 4 strains of C. botulinum type E, irradiated at 350 Krad produced toxin after 27 days at $40^{\circ}F$. Shucked soft-shelled clams inoculated with 10^4 and 10^6 mixed spores/g of the same 4 strains of type E, irradiated at 600 Krad produced toxin after 90 and 47 days respectively at $40^{\circ}F$.

Johnson et al. (24) showed evidence which indicated that serological procedures were applicable to the detection of botulinal toxins in food.

MATERIALS AND METHODS

I. Preliminary Research

Preparation of Shrimp and Shucked Oyster Homogenates

Fresh random samples of headless shrimp and shucked oysters were weighed out in triplicate 20 gram portions into 3 individual sterile screw cap Waring Blendor jars. To each Waring Blendor jar, 180 ml of Butterfields's Buffer were added and the mixture was blended for 2 minutes. The 1:10 shrimp homogenate was placed into a 16 ounce nalgene jar.

Inoculation of Shrimp and Shucked Oyster Homogenates with Spores

A mixed culture of C. botulinum type E spores was added to the 1:10 shrimp homogenate in 16 ounce nalgene screw top jars yielding a final concentration of 10^5 spores/g. The mixed culture of C. botulinum type E spores of 10^5 spores/g was determined by anaerobic plate counts on Liver Veal Agar (Difco) at 85°F after 5 days just prior to inoculation.

Clostridial Count

Immediately after irradiation, subsequent dilutions were made from the original 1:10 shrimp and shucked oyster homogenates. One ml of the ten fold dilutions were inoculated into Liver Veal 4% Egg Yolk Agar, Liver Veal Agar, Eugon Agar and Anaerobic Agar. Clostridial counts were made after incubation of the four culture media in a

Case Anaerobe Jar under a N_2 atmosphere and aerobically for 48 hours at 30°C.

Inoculation of Tryptone Glucose Peptone Medium with Spores

Triplicate sterile 25 x 150 mm tubes containing 30 ml of Tryptone Glucose Peptone Medium (T.G.P.) were inoculated with a mixed culture of C. botulinum type E yielding a final concentration of 10^5 spores/g.

Heat Treatment

Inoculated T.G.P. tubes at 10^5 spores/g were heat shocked in a wated bath for 10, 13 and 15 minutes at 145°F.

Clostridial Count from T.G.P.

Immediately after heat shocking, ten fold dilutions were made using Butterfields's Buffer. One ml of the ten fold dilutions were inoculated into Liver Veal Agar 4% Egg Yolk. Clostridial counts were made after incubation in a Case Anaerobe Jar under a N_2 atmosphere and aerobically for 48 hours at 30°F.

II. Assay of Shrimp and Oysters for C. botulinum, type E toxin

Handling Procedure for Shrimp and Oysters

The samples for this work were obtained from various commercial processors and packers in the Gulf Coast area.

The samples were packed in crushed ice in Arctic hampers and returned to the Department of Food Science and Technology Laboratories at L.S.U. in Baton Rouge within 5 hours. These samples had been out of the water 24 to 72 hours prior to arrival on campus.

The commercial frozen peeled, deveined and breaded samples obtained from retail outlets were also packed in crushed ice in Arctic hampers and returned to the laboratory. The samples were then assayed for C. botulinum type E according to the procedures recommended and used by the U. S. Department of Health, Education and Welfare, Food and Drug Administration (26).

Preparation of Shrimp Sample and Dilution

Random samples of whole or headless shrimp were weighed out in triplicate 20 gram portions into 3 individual sterile screw cap Waring Blendor jars. To each Waring Blendor jar, 180 ml of Butterfields's Phosphate Buffer were added and the mixture was blended for 2 minutes.

Preparation of Oyster Sample and Dilution

Random samples of fresh shucked oysters were weighed in triplicate 50 gram portions into 3 individual sterile screw cap Waring Blendor jars. To each Waring Blendor jar, 50 ml of Butterfields's Phosphate Buffer were added and the mixture was blended for 2 minutes.

Alcohol Treated Shrimp or Oysters

One ml samples were taken from the original dilution and added to 1 ml of 95% ethyl alcohol in sterile test tubes. This mixture was allowed to stand at room temperature for exactly 1 hour with occasional mixing.

After 1 hour of the ethyl alcohol treatment, sterile petri dishes of Liver Veal Agar 4% Egg Yolk Medium (L.V.E.Y.) and Liver Veal Agar

without egg yolk medium (L.V.) were streaked with samples of the alcohol treated suspension. These L.V.E.Y. streaked plates were incubated anaerobically in a Case Anaerobe Jar under a N_2 atmosphere, and L.V. streaked plates were incubated aerobically for 24 to 48 hours at 30°C.

Triplicate sterile 25 x 150 mm tubes containing 30 ml of Tryptone Glucose Peptone Medium (T.G.P.) were inoculated with 0.1 ml of the alcohol treated suspension. Also triplicate sterile 25 x 150 mm tubes containing 30 ml of Cooked Meat Medium (C.M.) were inoculated with 0.1 ml of the alcohol treated sample. The inoculated tubes were then incubated at 30°C for 10-12 days.

Non-Alcohol Treated Shrimp

Triplicate sterile tubes of T.G.P. were inoculated with 4 ml of the original 1:10 dilution of blended shrimp. Also triplicate sterile tubes of C. M. were inoculated with 4 ml of the original 1:10 dilution of blended shrimp.

At the same time, 10 ml of the original dilution of shrimp were added to 90 ml of sterile Butterfields's Phosphate Buffer resulting in a 100-fold dilution of the original dilution of blended shrimp. Triplicate sterile tubes of T.G.P. Medium were inoculated with 4 ml of this 100 fold dilution of shrimp. The inoculated tubes were incubated at 30°C for 10-12 days.

Non-Alcohol Treated Oysters

Triplicate sterile tubes of T.G.P. were inoculated with 4 ml of the original 1:1 dilution of blended oysters. Triplicate sterile tubes of C.M. were inoculated with 4 ml of the original 1:1 dilution

of blended oysters.

At the same time, 20 ml of the original homogenate of oysters were added to 80 ml of sterile Butterfields's Phosphate Buffer resulting in a 100-fold dilution of the original dilution of blended oysters. Triplicate sterile tubes of T.G.P. Medium were inoculated with 4 ml of the 100-fold dilution of oysters. Also triplicate sterile tubes of C.M. Medium were inoculated with 4 ml of the 100-fold dilution of oysters. The inoculated tubes were incubated at 30°C for 10-12 days.

Further Culturing

If growth occurred in the alcohol treated C.M. Medium or T.G.P. Medium, positive cultures were streaked on L.V.E.Y. Medium and L.V. Medium and incubated in a Case Anaerobe Jar under a N_2 atmosphere and aerobically, respectively for 24-48 hours at 30°C.

Suspected colonies with cultural and morphological characteristics were selected and gram stained for microscopic examination. If the organism appeared to be C. botulinum, a toxin assay was performed.

III. Inoculation Studies with Mixed Spore Suspension of C. botulinum Type E

Preparation of Spore Suspension

A mixed spore stock culture of C. botulinum type E consisting of Beluga, Alaska, Minneapolis and 8E strains were utilized throughout the experiment. These strains were provided by Dr. Schmidt of the Continental Can Company, Chicago, Illinois. The mixed C. botulinum type E spore suspensions were prepared according to Schmidt et al. (50). Stock cultures of C. botulinum type E were inoculated into

30 ml quantities in 25 x 150 mm screw cap tubes of trypticase peptone glucose (T.G.P.) sporulating medium and the suspension was heat shocked at 145°F for 10 minutes. These cultures were then incubated at 85°F for 24 hours and 10 ml quantities were inoculated into 8 ounce screw cap prescription bottles containing 200 ml of T.G.P. and incubated at 85°F for 5 days. The spores were then harvested by centrifugation at 5,000 rpm and washed twice with 0.85% sterile sodium chloride solution. For all strains, the concentration of spores obtained in this manner was approximately 1 x 10^6 per ml. The spores were then resuspended in sterile 0.85% sodium chloride solution to give the desired mixed spore stock concentration of 10^3 and 10^4 spores/g and held under refrigerated storage at 40°F.

Inoculation of Shrimp with Spores

A mixed culture of C. botulinum type E spores was added to 20 g of fresh headless shrimp in 16 ounce Nalgene screw top jars yielding a final concentration of 10^3 and 10^4 spores/g. The mixed culture of C. botulinum type E spores of 10^3 and 10^4 spores/g were determined by anaerobic plate counts on Liver Veal Agar (Difco) at 85°F after 5 days just prior to inoculation.

Inoculation of Shucked Oysters with Spores

A mixed culture of C. botulinum type E spores was added to 50 g of fresh shucked oysters in 16 ounce Nalgene screw top jars yielding a final concentration of 10^3 and 10^4 spores/g. The mixed culture of C. botulinum type E spores of 10^3 and 10^4 spores/g was determined by anaerobic plated counts on Liver Veal Agar (Difco) at 85°F after 5 days prior to inoculation.

Irradiation of Shrimp and Shucked Oysters

The inoculated shucked oysters and shrimp which were to be irradiated in 16 ounce Nalgene jars were placed inside a 21 x 11 inch diving bell and lowered to the bottom of a 20 foot well filled with water and allowed to remain in proximity to a 11,000-Curie source of the Co^{60} irradiator located at the Louisiana State University Nuclear Science Center. The dosages employed were 150, 200, 300, 500 and 600 Krads, respectively. Fricke dosimetry (1) was utilized for measuring gamma radiation by oxidation of ferrous ammonium sulfate and determination of the ferric ion spectrophotometrically. The average dose in the center of the diving bell was found to be 2,000 rads per minute.

Toxin Assay Procedure on Shucked Oysters and Shrimp

A modification of the Duff et al. (15) toxin assay procedure was utilized, in which 50 ml of sterile 0.85% sodium chloride was added to a 50 g sample of inoculated shucked oysters and homogenized 2 minutes in a sterile screw cap Waring Blendor giving a 1:1 dilution. In contrast the procedure for shrimp called for adding 20 ml of 0.85% sodium chloride to a 20 g sample of inoculated shrimp. A 10 ml aliquot of this dilution was adjusted to pH 6.2 using 1N NaOH or 1N HCl. Two milliliters of the former aliquot were transferred to two 16 x 125 mm screw cap tubes; one tube was mixed with 0.2 ml of a 10% solution of trypsin (Difco, 1:250 activity) resulting in a 1% trypsin solution. Trypsin digestion was carried out for 45 minutes at 98°F. Trypsin (0.2 ml) was added to the second tube and the tube then heated for 11 minutes at 212°F in boiling water. A 1:10 dilution of trypsinized mixtures of tubes 1 and 2 were prepared by adding 8 ml of

gel-phosphate buffer, pH 6.2. Two Carworth C.F.W. mice were each injected with 0.5 ml of type E antitoxin (5 international units). The white male mice utilized throughout the experiment were obtained from Carworth, Inc., New City, Rockland County, New York. The antitoxin utilized was obtained from the Biological Reagents Section, Communicable Disease Center, Atlanta, Georgia (3). After 30 minutes 0.5 ml of the nonheated trypsinized 1:10 dilution was injected into each protected animal. Two additional mice were each injected with 0.5 ml of nonheated trypsinized 1:10 extract. Another pair of mice was injected with 0.5 ml of the 1:10 heated trypsinized extract. The inoculated mice were maintained for 48 hours and observations were recorded at frequent intervals. These entire mice tests were performed in triplicate.

Type E toxin was considered to be present in the samples only when mice injected with the trypsinized sample died with symptoms of botulism within 24 hours as correlated with survival of mice protected with type E antitoxin without apparent illness for at least 48 hours as well as the control mice inoculated with the heated trypsinized samples.

RESULTS AND DISCUSSION

Clostridium botulinum in food or samples from nature has been shown by Dolman (10), Johannsen (20), and Meyer (32). There are only a few media for the isolation of C. botulinum, therefore selecting the best medium is of great importance.

Isolation tests for demonstration of C. botulinum, type E toxin were performed utilizing the methods of Slocum (53) and Schmidt (47, 48) as well as the latest methods of the U. S. Food and Drug Administration, Department of Health, Education and Welfare (7).

The procedure employed for demonstration of C. botulinum usually involves the examination for the presence of the toxin from an enriched broth culture. Dolman (10) stated that demonstration of the toxin is sufficient evidence that C. botulinum is present; however, this evidence is strengthened by isolation of the organism in pure culture.

Johannsen (20) stated that the failure to find toxin in an enriched broth culture does not necessarily indicate the complete absence of C. botulinum from the original sample, since other organisms may inhibit its growth or destroy the toxin.

Due to the presence of nontoxigenic strains of C. botulinum type E toxin in nature, it was decided to isolate C. botulinum type E toxin in pure culture from alcohol treated and nontreated shrimp and oysters samples on T.G.P. enrichment broth, followed by mouse toxicity tests.

Comparison of Four Different Media for Recovery of Spores

The results of Tables I and II were taken from an average of triplicate determinations. Tables I and II showed that Liver Veal 4% Egg Yolk Agar was a superior medium for recovery of C. botulinum, type E after irradiation at 300 and 600 Krads as well as for the non-irradiated sample. Nickerson and Goldblith (39) found that Liver Veal 4% Egg Yolk Agar, which is used by the U. S. Food and Drug Administration, to be the medium of choice for recovery of C. botulinum, type E, when compared to several other different mediums.

The results of Table III were taken from an average of triplicate determinations. Table III showed heat shocking of mixed spores for 10, 13 and 15 minuted decreased the number of viable spores as compared to the non-heated shocked spores. A decision was made not to use a heat pretreatment before isolating C. botulinum, type E from shrimp and oysters. The reasoning was based on the lower heat resistance of C. botulinum, type E than all other C. botulinum types and the fact that there might be small numbers of the organism in shrimp and oysters and thereby lessen the chances of recovery.

Segner et al. (52) showed in thermal resistance studies in laboratory media that the Minneapolis strain was the most resistant and Alaska the least resistant. The thermal resistance of the 8E strain was about 80% of that of the Minneapolis and the Alaska strain was 50%.

Because of the strong evidence in support of the culture media for C. botulinum type E from the literature, plus 3 years of personal experience, T.G.P. broth and Liver Veal Agar 4% Egg Yolk were used throughout these investigations.

Segner et al. (52) studied the effect of salt (sodium chloride) concentration on the outgrowth of type E spore inocula in laboratory culture media. The investigators reported that in a laboratory culture medium C. botulinum type E possessed a level of sodium chloride inhibition considerably below that recognized for types A and B. At 60°, 70°, and 85°F, 5.0% salt was required for complete inhibition of spore outgrowth. Lower temperatures of incubation had little effect on the salt tolerance of type E spore inocula. Salt at a concentration of 4.5% was required for definite inhibition at 46° and 50°F.

Segner et al. (52) reported the effect of pH on the outgrowth of type E spore inocula at various temperatures. They found that type E, C. botulinum would not tolerate any lower pH (5.03 to 5.46) than that accepted as completely inhibitory for outgrowth of types A and B spores.

Assay for C. botulinum Type E in Shrimp

The results of the microbiological assay for C. botulinum type E on Gulf Coast fresh shrimp samples obtained from commercial processors, as well as frozen samples from retail outlets are found in the following table. Table IV presents the results of assays on Louisiana whole and headless fresh Gulf shrimp, as well as the results of assays on retail fresh whole shrimp samples and frozen peeled and deveined shrimp. Table IV presents the results of assays on headless fresh shrimp samples from commercial processors and frozen breaded, peeled and deveined shrimp samples from retail outlets in Texas. Table IV presents the results of assays on Alabama and Mississippi

fresh headless shrimp samples from commercial processors and retail breaded, peeled and deveined frozen shrimp samples. Table IV also presents the results of assays on fresh headless shrimp samples from commercial processors and frozen breaded, peeled and deveined shrimp samples from retail outlets in Georgia. Finally, Table IV shows the results of assays on Florida frozen headless, breaded, peeled and deveined shrimp samples which were obtained from commercial processors and also frozen breaded shrimp samples from a retail outlet.

These results were based on at least two different samples from each location. Each sample was run in triplicate. Over 700 pounds of whole, headless fresh shrimp and frozen peeled and deveined commercial frozen products were sampled and examined in triplicate and no C. botulinum, type E toxin was found present.

Assay for C. botulinum Type E in Oysters

The results of the microbiological assay for C. botulinum type E toxin on Gulf Coast fresh oysters, washed, unwashed and breaded frozen samples obtained from commercial processors, as well as frozen samples are found in Table V. Table V presents the results of assays on Louisiana fresh washed and unwashed shucked oysters obtained from commercial processors, as well as results of assays on retail fresh shucked oyster samples. Table V shows results on washed fresh shucked oysters from commercial processors in Mississippi and Alabama.. Table V presents the results of assays on washed frozen shucked oysters obtained from retail outlets in Alabama. Table V also shows the results of assays on frozen breaded and canned oysters

obtained from retail outlets in Georgia. Table V presents also the results of assays on Texas unwashed fresh shucked oysters and breaded frozen oysters from commercial processors.

The results of Table V were based on at least two different samples from each location. Each sample was run in triplicate.

Over 300 pounds of fresh whole washed and unwashed oysters and frozen breaded retail oysters were sampled and examined in triplicate and no C. botulinum type E toxin was detected.

In the present investigation, a total of more than 1,000 pounds of Gulf Coast fresh shrimp and oyster samples as well as frozen commercial samples in triplicate has been found negative for C. botulinum type E toxin.

The only single Clostridium present and identified was C. sporogenes in a shrimp sample from a retail outlet in New Orleans, Louisiana.

According to U. S. Public Health records there have not been any cases of botulism, specifically type E, associated with Gulf Coast shrimp or oysters. A review of the literature and state public health records has never revealed a single case of C. botulinum, type E which has been directly traced to fresh Gulf Coast shrimp and oysters. However, Ward (55) very recently found type E present in 1 of the 2 samples of white shrimp from Aransas Pass, Texas (1 tube of 10 showed the presence of type E). According to U. S. Public Health Laboratory at Dauphin Island, one oyster sample from Mobile Bay from an area of low salt concentration has been found positive for C. botulinum type E (54).

These reported findings recently of C. botulinum, type E toxin

emphasize the low relative incidence of C. botulinum in the Gulf of Mexico. However, this does not negate the possible potential inherent danger of the presence of C. botulinum, type E toxin.

Johnston et al. (23) showed that the chemical resistance of type E spores could be utilized to separate them from the contaminating vegetative microflora. They found that a 50% concentration of ethanol was germicidal for vegetative cells, without adversely affecting type E spores. This concentration was obtained by mixing equal parts of absolute ethanol and the enrichment culture. Further work by Johnston et al. (23) showed that when enrichment cultures from fish were streaked on media without alcohol treatment, the overgrowth of facultative microorganisms prevented the recovery of C. botulinum type E. These workers also demonstrated that direct plating of macerated fish without alcohol treatment was not successful for isolating the species, whereas alcohol treatment of the fish often permitted isolation by direct plating. They felt that the ability of C. botulinum type E to sporulate more readily than many other sporeformers in T.G.P. enrichment broth aided in its isolation by means of the alcohol treatment.

Toxicity Tests on Gulf Coast Headless Shrimp

The results of Tables VI and VII show that no C. botulinum, type E toxin was produced when fresh shrimp samples were stored on ice ($33^{o}F$) up to 14 days, after being inoculated with mixed C. botulinum type E spore suspensions of 10^{3} and 10^{4} spores/g and exposed to Co^{60} radiation from 150 to 500 Krads.

In Tables VI and VII, no toxin production was demonstrated when

fresh nonirradiated samples were stored on ice ($33^{o}F$) after 30 and 31 days, after being inoculated with C. botulinum type E spores at 10^{3} and 10^{4} spores/g.

In Tables VIII and IX toxin production was indicated after 7 and 14 days when fresh shrimp were stored at $42^{o}F$ after being inoculated with mixed C. botulinum type E spores at 10^{3} and 10^{4} spores/g and exposed to Co^{60} radiation at 150, 200, and 300 Krads as well as in the nonirradiated control.

In Tables VIII and IX C. botulinum, type E toxin production was shown after 30 and 31 days when fresh shrimp were stored at $42^{o}F$ after being inoculated with mixed C. botulinum type E spores at 10^{3} and 10^{4} spores/g respectively and subjected to Co^{60} radiation at 500 Krads.

It should be noted in Tables VIII and IX that at $42^{o}F$ with an inoculation of 10^{3} and 10^{4} spores/g respectively at 500 Krads, no toxicity was found except after 30 and 31 days.

It thus appears that there is a temperature requirement for the production of toxin by type E C. botulinum as no toxin was produced in the irradiated or controlled fresh shrimp stored at 32^{o} F (ice) during the 30 days of these tests.

Toxicity Tests on Gulf Coast Shucked Oysters

The results of Tables VII and VIII show that no C. botulinum, type E toxin was produced when fresh Gulf Coast shucked oysters were stored on ice ($33^{o}F$) up to 30 days, after being inoculated with mixed C. botulinum type E spores of 10^{3} and 10^{4} spores/g and exposed to Co^{60} radiation at 150, 200, 300 and 500 Krads.

It should be noted in Tables X and XI at $33^{o}F$ with 10^{3} and 10^{4}

spores/g that no toxicity was found on nonirradiated Gulf Coast shucked oysters up to 31 days storage time.

In Tables XII and XIII C. botulinum, type E toxin production was indicated after 30 days when fresh Gulf Coast shucked oysters were stored at 42°F after being inoculated with mixed C. botulinum type E spores at 10^3 and 10^4 spores/g and subjected to Co^{60} radiation at 150, 200, 300, and 500 Krads as well as in the nonirradiated control.

It thus appears that there is a temperature requirement for the production of toxin by type E C. botulinum as no toxin was produced in the irradiated or control fresh oysters stored at 32°F (ice) during the 31 days of these tests.

Nickerson and Goldblith (39) reported that C. botulinum type E was found in Chesapeake Bay clams in very small numbers.

The fact that C. botulinum type E produced toxin in 7 days in headless shrimp while the organism took 30 days in shucked oysters indicated that shrimp appeared to be a better nutrient medium for growth and toxin production than shucked oysters at 42°F.

It should be noted that when fresh Gulf Coast headless shrimp were stored at 42°F after being inoculated with mixed C. botulinum type E spores at 10^3 and 10^4 spores/g and subjected to Co^{60} radiation at 500 Krads, no toxicity was found up to 14 days. This suggests that the 500 Krad dose retarded the natural ability of the organism in the spore stage to germinate into the vegetative stage and produce toxin. Another possibility is that the delayed toxin production at 500 Krads in headless shrimp may be due to toxic products or inhibitors produced in the shrimp which might have interfered with the spore germination or vegetative cell formation.

Toxin production was found in nonirradiated and irradiated shucked oysters inoculated with mixed spores suspensions of 10^3 and 10^4 spores/g of C. botulinum type E after 30 days at 42°F. Nickerson and Goldblith (39) demonstrated similar toxin production in non-irradiated and irradiated Haddock and shucked clams inoculated with mixed spores of C. botulinum type E at 10^2 and 10^4 spores/g.

Regular checks by plate counts after inoculation and toxin production for C. botulinum, type E were performed on both irradiated and control shrimp and oyster samples. These plate counts indicated only a small increase in number from the original spore inocula. Thus it appears that toxin production was due to sporulation followed by the vegetative stage and subsequent toxin production from the original inocula rather then the small increase in the population size.

Liuzzo and Novak (30) stated that shrimp storage life could be extended from 21 to 37 days when irradiated at 50,000 rads and stored at 32°F. The bacterial count showed that irradiation resulted in a bacterial count of 40,000 as compared to 800,000 for the non-irradiated samples when they were stored at 32°F for the same period of time. It was also evident that storage at 32°F was more beneficial than 36-40°F in keeping the bacterial count low.

Novak et al. (41) showed that oysters irradiated with 0.2 Mrad and stored in crushed ice for 7, 14, and 21 days were superior in flavor, odor, and appearance to unirradiated samples after 7 days' storage, and still acceptable after 21 days. Irradiation reduced initial bacteria (total plate) counts by 99%. Trimethylamine and ammonia increased more rapidly in unirradiated oysters than in

irradiated oysters.

Bacteria (total plate) counts in shrimp and oysters can be reduced significantly by low dose gamma radiation. C. botulinum type E will produce toxin in shrimp and oysters at 42°F but not on ice (33°F). This definitely indicated that shellfish should be stored on ice to assure against the growth of C. botulinum, type E toxin as well as all other bacteria for maximum consumer acceptance and safety.

It was also noted in fresh shrimp that the pH increased toward the alkaline range during iced storage. Nickerson and Goldblith (39) found in investigations with fish juice that type E, C. botulinum toxin was much less heat stable at higher (alkaline) pH levels.

In fresh shrimp, control and experimental samples, the original pH was approximately 7 and increased steadily to pH 8.5 at the end of 30 days and remained relatively constant at this pH thereafter. It was determined experimentally by tests with alkali in the laboratory that between pH 8.5 and 9.5 complete inactivation of the toxin of C. botulinum, type E was obtained in relatively short periods of time utilizing the mouse assay. There was very little difference in this pH pattern between the irradiated and control samples at 32°F and 42°F. This natural safety factor when coupled with the observation that shrimp stored at 32°F and 42°F become organoleptically unacceptable in regard to spoilage (bad odor, flavor, and texture) before toxin production at 42°F supports the evidence for the absence of toxin in the survey for the presence of type E toxin in Gulf Coast iced fresh commercial shrimp.

In fresh iced oysters, the original pH was approximately 6.4 and decreased to pH 5.2 within 2 weeks and then gradually rose to pH 6.0

by the end of 30 days and remained relatively constant thereafter. In contrast to the fresh iced shrimp, there was a difference in the pH pattern between the irradiated and control iced oyster samples. The irradiated fresh iced oysters as well as those stored at 42°F dropped to a lower pH of 3.5 in two weeks and rose very little by the end 30 days and remained relatively constant thereafter. The nonproduction of toxin at 32°F and its production only after 30 days in the irradiated and control inoculated samples may well be associated with this low non-physiological pH. This finding is in agreement with the findings of Segner et al. (52) who reported that pH's lower than 5 are completely inhibitory for C. botulinum, type E spore outgrowth. After 30 days both irradiated and control samples of iced fresh oysters and fresh oysters stored at 42°F are organoleptically unacceptable in regard to odor, appearance and free liquor when type E toxin was first detected at 42°F only.

In the United States most shrimp are cooked before being consumed. This serves as an additional safety factor against the effect of botulinum toxin if present. However, some oysters are eaten raw, but they are also consumed roasted, fried, and boiled. If shellfish are cooked properly, all six types of botulinum toxins will be destroyed by heat. Cooking for 10 minutes at 100°C will destroy all known C. botulinum toxins present.

Table I. Irradiation Resistance of Mixed Spores of Type E* C. botulinum in a 1:10 Butterfields's Buffer Homogenate of Gulf Coast Headless Shrimp and the Effect on Recovery in Solid Culture Medium.

Culture Medium	Clostridial Count Per Gram and Irradiation Dose		
	0 Krad	300 Krad	600 Krad
Liver Veal 4% Egg Yolk Agar	5.2×10^5	5.2×10^3	2.4×10^3
Liver Veal Agar	3.9×10^5	3.9×10^3	1.2×10^3
Eugon Agar	3.1×10^5	4.8×10^3	1.3×10^3
Anaerobic Agar	2.8×10^5	3.6×10^3	1.2×10^3

*Beluga, Alaska, Minneapolis and 8E.

Table II. Irradiation Resistance of Mixed Spores of Type E* C. botulinum in a 1:10 Butterfields's Buffer Homogenate of Gulf Coast Shucked Oysters and the Effect on Recovery in Solid Culture Medium.

Culture Medium	Clostridial Count Per Gram and Irradiation Dose		
	0 Krad	300 Krad	600 Krad
Liver Veal 4% Egg Yolk Agar	4.8×10^5	5.8×10^3	2.9×10^3
Liver Veal Agar	3.8×10^5	4.2×10^3	1.9×10^3
Eugon Agar	3.3×10^5	4.5×10^3	2.2×10^3
Anaerobic Agar	2.5×10^5	3.9×10^3	1.8×10^3

*Beluga, Alaska, Minneapolis and 8E.

Table III. Effect of Heat Shocking and Non-Heat Shocking on Mixed Spores of Type E* C. botulinum inoculated at 10^5 Spores/g in Tryptone Glucose Peptone Medium.

Treatment	Clostridial Count Per Gram on Liver Veal Agar 4% Egg Yolk
Non-heat shocked	5.2×10^5
Heat shocked (10 min. at 145°F)	5.0×10^5
Heat shocked (13 min. at 145°F)	4.9×10^4
Heat shocked (15 min. at 145°F)	4.0×10^4

*Beluga, Alaska, Minneapolis and 8E.

Table IV. Assay for C. botulinum Type E in Shrimp

Description of Sample	No. of Samples	State of Origin	C. botulinum Type E
Fresh whole	9	Louisiana	none
Fresh headless	15	Louisiana	none
Frozen peeled, deveined	4	Louisiana	none
Fresh headless	3	Texas	none
Frozen peeled, deveined	2	Texas	none
Frozen breaded	3	Texas	none
Fresh headless	3	Miss. and Alabama	none
Frozen peeled, deveined	2	Miss. and Alabama	none
Frozen breaded	2	Miss. and Alabama	none
Fresh headless	2	Georgia	none
Frozen peeled, deveined	2	Georgia	none
Frozen breaded	2	Georgia	none
Fresh headless	4	Florida	none
Frozen headless	7	Florida	none
Frozen peeled, deveined	12	Florida	none
Frozen breaded	5	Florida	none

Table V. Assay for C. botulinum Type E in Oysters

Description of Sample	No. of Samples	State of Origin	C. botulinum Type E
Fresh washed	10	Louisiana	none
Fresh unwashed	6	Louisiana	none
Fresh washed	5	Miss. and Alabama	none
Fresh washed	7	Alabama	none
Frozen breaded	2	Georgia	none
Frozen canned	2	Georgia	none
Fresh unwashed	3	Texas	none
Frozen breaded	2	Texas	none

Table VI. Toxicity tests on Gulf Coast headless shrimp inoculated with a mixed culture of 4 strains of C. botulinum type E* at 10^3 spores/g. All samples were stored on ice (33°F.)

Sample No.	Dose in Krad.	Storage Time in Days	Toxicity
1	0	0	Negative - no death**
2	150	0	" "
3	200	0	" "
4	300	0	" "
5	500	0	" "
1	0	7	" "
2	150	7	" "
3	200	7	" "
4	300	7	" "
5	500	7	" "
1	0	14	" "
2	150	14	" "
3	200	14	" "
4	300	14	" "
5	500	14	" "
13	0	30	" "

*Beluga, Alaska, Minneapolis, and 8E.

**Negative denotes survival, as mice did not die when injected with 1:10 dilution of trypsinized sample correlated with control and protected mice.

Table VII. Toxicity Tests on Gulf Coast headless shrimp inoculated with a mixed culture of 4 strains of C. botulinum type E* at 10^4 spores/g. All samples were stored on ice (33°F.).

Sample No.	Dose in Krad.	Storage Time in Days	Toxicity
1	0	0	Negative - no death**
2	150	0	" "
3	200	0	" "
4	300	0	" "
5	500	0	" "
1	0	7	" "
2	150	7	" "
3	200	7	" "
4	300	7	" "
5	500	7	" "
1	0	14	" "
2	150	14	" "
3	200	14	" "
4	300	14	" "
5	500	14	" "
14	0	31	" "

*Beluga, Alaska, Minneapolis, and 8E.

**Negative denotes survival, as mice did not die when injected with 1:10 dilution of trypsinized sample correlated with control and protected mice.

Table VIII. Toxicity Tests on Gulf Coast headless shrimp inoculated with a mixed culture of 4 strains of C. botulinum type E* at 10^3 spores/g. All samples were stored at 42°F.

Sample No.	Dose in Krad.	Storage Time in Days	Toxicity
1	0	0	Negative - no death**
2	150	0	" "
3	200	0	" "
4	300	0	" "
5	500	0	" "
1	0	7	Positive - death***
2	150	7	" "
3	200	7	" "
4	300	7	" "
5	500	7	Negative - no death
1	0	14	Positive - death
2	150	14	" "
3	200	14	" "
4	300	14	" "
5	500	14	Negative - no death
15	500	30	Positive - death

*Beluga, Alaska, Minneapolis and 8E.

**Negative denotes survival, as mice did not die when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

***Positive denotes no survival, as mice died when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

Table IX. Toxicity Tests on Gulf Coast headless shrimp inoculated with a mixed culture of 4 strains of C. botulinum type E* at 10^4 spores/g. All samples were stored at 42°F.

Sample No.	Dose in Krad.	Storage Time in Days	Toxicity
1	0	0	Negative - no death**
2	150	0	" "
3	200	0	" "
4	300	0	" "
5	500	0	" "
1	0	7	Positive - death***
2	150	7	" "
3	200	7	" "
4	300	7	" "
5	500	7	Negative - no death
1	0	14	Positive - death
2	150	14	" "
3	200	14	" "
4	300	14	" "
5	500	14	Negative - no death
16	500	31	Positive - death

*Beluga, Alaska, Minneapolis and 8E.

**Negative denotes survival, as mice did not die when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

***Positive denotes no survival, as mice died when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

Table XI. Toxicity Tests on Gulf Coast shucked oysters inoculated with a mixed culture of 4 strains of C. botulinum type E* at 10^3 spores/g. All samples were stored on ice (35°F.).

Sample No.	Dose in Krad.	Storage Time in Days	Toxicity
6	0	0	Negative - no death**
7	150	0	" "
8	200	0	" "
9	300	0	" "
10	500	0	" "
6	0	15	" "
7	150	15	" "
8	200	15	" "
9	300	15	" "
10	500	15	" "
6	0	30	" "
7	150	30	" "
8	200	30	" "
9	300	30	" "
10	500	30	" "
11	0	31	" "

*Beluga, Alaska, Minneapolis and 8E.

**Negative denotes survival, as mice did not die when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

Table XI. Toxicity Tests on Gulf Coast shucked oysters inoculated with a mixed culture of 4 strains of C. botulinum type E* at 10^4 spores/g. All samples were stored on ice (35°F.).

Sample No.	Dose in Krad.	Storage Time in Days	Toxicity
6	0	0	Negative - no death**
7	150	0	" "
8	200	0	" "
9	300	0	" "
10	500	0	" "
6	0	15	" "
7	150	15	" "
8	200	15	" "
9	300	15	" "
10	500	15	" "
6	0	30	" "
7	150	30	" "
8	200	30	" "
9	300	30	" "
10	500	30	" "
11	0	31	" "

*Beluga, Alaska, Minneapolis and 8E.

**Negative denotes survival, as mice did not die when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

Table XII. Toxicity Tests on Gulf Coast shucked oysters inoculated with a mixed culture of 4 strains of C. botulinum type E* at 10^3 spores/g. All samples were stored at 42°F.

Sample No.	Dose in Krad.	Storage Time in Days	Toxicity
6	0	0	Negative - no death**
7	150	0	" "
8	200	0	" "
9	300	0	" "
10	500	0	" "
6	0	15	" "
7	150	15	" "
8	200	15	" "
9	300	15	" "
10	500	15	" "
6	0	30	Positive - death***
7	150	30	" "
8	200	30	" "
9	300	30	" "
10	500	30	" "
11	0	31	" "

*Beluga, Alaska, Minneapolis and 8E.

**Negative denotes survival, as mice did not die when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

***Positive denotes no survival, as mice died when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

Table XIII. Toxicity Tests on Gulf Coast shucked oysters inoculated with a mixed culture of 4 strains of C. botulinum type E* at 10^4 spores/g. All samples were stored at 42°F.

Sample No.	Dose in Krad.	Storage Time in Days	Toxicity
6	0	0	Negative - no death**
7	150	0	" "
8	200	0	" "
9	300	0	" "
10	500	0	" "
6	0	15	" "
7	150	15	" "
8	200	15	" "
9	300	15	" "
10	500	15	" "
6	0	30	Positive - death***
7	150	30	" "
8	200	30	" "
9	300	30	" "
10	500	30	" "
11	0	31	" "

*Beluga, Alaska, Minnea[illegible], and 8E.

**Negative denotes survival, as mice did not die when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

***Positive denotes no survival, as mice died when injected with a 1:10 dilution of trypsinized sample correlated with control and protected mice.

SUMMARY

1. A survey of Gulf Coast commercially handled shrimp and oysters has indicated the absence of C. botulinum type E toxin.
2. No toxin was produced when fresh Gulf Coast headless shrimp and shucked oysters were stored on ice ($33^{\circ}F$) up to 30 days, after being inoculated with mixed C. botulinum type E spores at 10^3 and 10^4 spores/g and either without irradiation or with exposure to Co^{60} radiation at 150, 200, 300, and 500 Krads.
3. Fresh Gulf Coast headless shrimp were inoculated with mixed C. botulinum type E spores at 10^3 and 10^4 spores/g. Samples were subjected to Co^{60} radiation at 150, 200, 300, and 500 Krads. The irradiated samples along with nonirradiated controls were then stored at $42^{\circ}F$. After 7 and 14 days, toxin was found in all samples except those which had received 500 Krads; in these, toxin was demonstrated after 30 and 31 days.
4. Toxin production was shown after 30 days when fresh shucked oysters were stored at $42^{\circ}F$ after being inoculated with mixed C. botulinum type E spores at 10^3 and 10^4 spores/g and subjected to Co^{60} radiation at 150, 200, 300 and 500 Krads along with a non-irradiated control.

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APPENDIX

Table I. Geographic Distribution of Verified Type E Botulism Outbreaks

Place of occurrence		Outbreaks	Cases	Deaths
Japan:	Hokkaido	29	222	42
	Northern Honshu	20	82	37
U. S.:	Alaska	7	19	6
	Other states	8	36	15
Canada:	British Columbia	8	20	11
	Labrador	3	10	8
Sweden		3	6	1
Denmark		2	7	0
U.S.S.R.		2	2	2
Totals		82	404	122

Figures from Dolman and Iida (13) supplemented by more recent data from Japan by Nakamura (38), and the Morbidity and Mortality Weekly Report (37).

Table 2. Total Cases of Botulism in the U.S.A. from 1899 through 1963 by Decade

STATES	1899-1909	1910-1919	1920-1929	1930-1939	1940-1949	1950-1959	1960-1963	TOTAL
California	11	104	87	105	109	50	12	478
Washington	0	23	43	46	30	15	8	165
Colorado	0	17	27	31	23	25	2	125
New Mexico	0	0	0	37	33	7	3	80
New York	0	18	21	19	12	0	2	72
Michigan	0	15	34	0	2	0	9	60
Oregon	0	3	18	20	12	3	0	56
Tennessee	0	0	7	15	6	5	15	48
Kentucky	0	0	0	11	11	11	10	43
Montana	0	7	5	17	3	2	0	34
North Dakota	0	0	0	21	9	0	3	30
Ohio	0	14	12	3	0	1	0	30
Wyoming	0	0	21	8	0	0	0	29
Nebraska	0	2	3	12	10	0	3	27
New Jersey	0	3	2	5	4	13	0	27
Idaho	0	4	2	8	0	6	3	23
Mississippi	0	0	0	0	4	17	0	21
Indiana	0	7	11	0	0	2	0	20
Pennsylvania	0	0	9	1	3	2	3	18
Illinois	0	4	4	0	2	6	0	16

Table 2. (Continued) Total Cases of Botulism in the U.S.A. from 1899 through 1963 by Decade

STATES	1899-1909	1910-1919	1920-1929	1930-1939	1940-1949	1950-1959	1960-1963	TOTAL
Utah	0	1	0	1	12	0	0	14
Minnesota	0	0	0	5	0	3	5	13
Alaska*	0	0	0	0	0	10	3	13
Texas	0	0	6	4	0	1	0	11
Arizona	0	0	5	0	4	2	0	11
Virginia	0	0	0	2	5	2	0	9
Florida	0	7	0	0	0	0	1	8
Massachusetts	0	6	0	2	0	0	0	8
Maryland	0	0	0	0	4	3	0	7
Alabama	0	0	3	0	1	0	3	7
South Dakota	0	0	0	5	2	0	0	7
Nevada	0	0	0	0	3	3	0	6
Wisconsin	0	3	2	1	0	0	0	6
Oklahoma	0	0	0	2	1	3	0	6
Connecticut	0	0	0	1	4	0	0	5
Iowa	0	5	0	0	0	0	0	5
Maine	0	0	4	0	0	0	0	4
West Virginia	0	0	0	0	3	0	1	4
Louisiana	0	0	0	0	0	3	0	4
Washington, D. C.	0	0	0	0	3	0	0	3

*Since 1950 only.

Source: U. S. Public Health Service, Communicable Disease Center (36)
Meyer and Eddie (34)

Table 2. (Continued) Total Cases of Botulism in the U.S.A. from 1899 through 1963 by Decade

STATES	1899-1909	1910-1919	1920-1929	1930-1939	1940-1949	1950-1959	1960-1963	TOTAL
Arkansas	0	0	0	2	0	0	1	3
Missouri	0	0	2	0	0	0	0	2
North Carolina	0	0	0	0	0	2	0	2
Georgia	0	0	0	0	1	0	0	1
Hawaii*	0	0	0	0	0	0	0	0
New Hampshire	0	0	0	0	0	0	0	0
Vermont	0	0	0	0	0	0	0	0
Rhode Island	0	0	0	0	0	0	0	0
Kansas	0	0	0	0	0	0	0	0
Delaware	0	0	0	0	0	0	0	0
South Carolina	0	0	0	0	0	0	0	0
TOTALS	11	243	328	384	316	197	82	1561

*Since 1950 only.

Source: U. S. Public Health Service, Communicable Disease Center (36)
Meyer and Eddie (34)

Table 3. Botulism Outbreaks Associated with Commercial Foods in the U.S.A.

YEAR	FOOD	OUTBREAK	CASES	DEATHS	TYPE
1906	Pork and Beans	1	3	3	-
1910	String Beans	1	4	4	-
1912	Clam Juice	1	2	1	-
1913	Clam Juice	1	3	2	-
1914	Clam Juice	1	2	2	-
1915	Tomato Catsup	1	2	0	-
	Sausage	1	2	2	-
1918	Corn	1	1	0	-
	Minced Olives	1	2	2	-
	Tuna	1	1	1	-
1919	Olives	3	28	17	A
	Summer Sausage	1	3	0	-
1920	Ripe Olives	1	7	7	A
	Ripe Olives	2	2	0	-
	Minced Olives	1	5	0	A
	Minced Olives	1	1	1	-
	Spinach	1	6	3	A
	Spinach	1	2	2	-
	Ham	1	4	4	-
	Milk	1	4	0	-
	Beets	1	5	5	B
1921	Spinach	3	32	4	A
	Ripe Olives	1	5	3	A
1922	Spinach	2	11	6	-
1924	Ripe Olives	2	13	6	-
	Ripe Olives	1	9	2	A
1925	Sardines	1	2	2	-
	Sardines	1	2	2	A
	Spinach	1	5	1	B
	Potted Meat	1	4	4	B
1929	Shallots	1	2	1	B
1931	Antipasto	1	3	1	-
	Milk	1	1	0	A
	Sardines	1	2	1	-
1934	Sprats	1	3	1	E
1936	Clams (Japanese Canned)	1	4	4	B
1938	Tuna	1	2	2	-
1941	Mushroom Sauce	1	3	1	E
1951	Cheese	1	1	1	-

Table 3. (Continued) Botulism Outbreaks Associated with Commercial Foods in the U.S.A.

YEAR	FOOD	OUTBREAK	CASES	DEATHS	TYPE
1960	Smoked Ciscos	1	2	2	E
1963	Tuna	1	3	2	E
	Smoked Whitefish	1	2	2	E
	Smoked Whitefish Chub	1	17	5	E
	Liver Paste	1	2	0	A
TOTALS		51	219	109	

Source: Meyer and Eddie (34)
U. S. Public Health Service Communicable Disease Center (36)

Table 4. Known Outbreaks of Type E Botulism

YEAR	STATE	FOOD	CASES	DEATHS
1932	New York	Smoked Salmon (Canadian origin)	3	1
1934	New York	Sprats (German origin)	3	1
1941	California	Mushroom Sauce	3	1
1950	Alaska (Point Hope)	Beluga Flippers	5	0
1952	Alaska (Selawik)	Beluga Flippers	1	1
1956	Alaska (Kotzebue)	Beluga Muktuk	3	2
1956	Alaska (Anchorage)	Beluga Muktuk	2	1
1959	Alaska (Hydaburg)	Stink Eggs (Salmon Eggs)	1	1
1959	Alaska (Scammon Bay)	Seal or Whale Flipper	7	1
1960	Alaska (Ketchikan)	Ketdukon Salmon	2	2
1960	Minnesota	Cisco-vacuum packed	2	2
1961	Washington	Uncooked Salmon Eggs	4	1
1963	Michigan	Tuna (California packed)	3	2
1963	Michigan	Smoked Whitefish	2	2
1963	Tennessee Alabama Kentucky	Smoked Whitefish Chubs (vacuum packed in Michigan)	17	5
TOTALS			58	23

Source: Dolman (12) and Meyer (33)

Table 5. Botulism in the U.S.A. in 1963

OUTBREAK	CASES	DEATHS	FOOD	TYPE	LOCATION	PROCESSOR
1	2	0	Chili Peppers	A	California	Non-commercial
2	2	0	Liver Paste	A	New York City	Commercial
3	1	1	Green Beans	B	West Virginia	Non-commercial
4	5	1	Corn	B	Kentucky	Non-commercial
5	2	1	Green Beans	B	Colorado	Non-commercial
6	3	0	Green Beans	B	Pennsylvania	Non-commercial
7	3	2	Tuna Fish	E	Michigan	Commercial
8	2	2	Smoked Whitefish	E	Michigan	Commercial
9	17	5	Smoked Whitefish Chubs	E	Tennessee, Alabama, Kentucky	Commercial
10	6	1	Mushrooms	Unknown	California	Non-commercial
11	1	0	Smoked Whitefish	Unknown	Minnesota	Non-commercial
12	2	1	Figs	Unknown	California	Non-commercial
TOTALS	46	14				

Source: Morbidity and Mortality Weekly Report (37)

Table 6. Botulism by Specific Type in the U.S.A. in 1963

	OUTBREAKS	CASES	DEATHS
Type A	2	4	0
Type B	4	11	3
Type E	3	22	9
Unknown	3	9	2
TOTALS	12	46	14

Source: Morbidity and Mortality Weekly Report (37)

Media

Difco Liver Veal Agar (9) 4% Egg Yolk

Fresh eggs were washed with a stiff brush, drained and then soaked in 70% ethyl alcohol for 30 minutes.

The eggs were cracked aseptically, the eggwhite was separated from the yolk and discarded leaving the yolk in the eggshell. The yolk was stabbed in the center with a sterile straight inoculating needle opening a hole approximately one fourth inch in diameter. The contents of the yolk sack were taken out by means of an open tip 10 ml pipette and drained into a sterile graduated cylinder. An equal volume of physiological saline was added to the volume of yolk and mixed gently until thoroughly mixed.

Eighty milliliters of egg yolk saline mixture was added to one liter of melted sterile Liver Veal Agar coiled to 45-50°C. The Liver Veal Egg Yolk medium was poured into petri dishes immediately then dried at room temperature for 2 or 3 days before being utilized.

Typticase-Glucose-Peptone Medium

1. Typticase (BBL)----------50 g
   Peptone (Difco)----------5 g
   Sodium Thioglycollate (BBL)----------2 g
   Glucose (Difco)----------4 g
   Distilled Water----------1000 ml
2. Typticase-Glucose-Peptone Medium was adjusted to pH 7.0 with 1N NaOH and 1N HCl and dispensed in 30 ml amounts in 25 x 150 mm screw cap tubes; autoclaved at 250°F for 10 minutes.

Difco Eugon Agar (9)

Difco Anaerobic Agar (9)

Difco Liver Veal Agar (9)

Difco Cooked Meat Medium (9)

Preparation of Physiological Saline

Sodium chloride (8.5 g) was added to 1 liter of distilled water.

Buffers

Gel-Phosphate

1. Gelatin (Difco)----------2 g
   Na_2HPO_4 (Merck)----------4 g
   Distilled water----------1000 ml
2. Adjusted to pH 6.2 using 1N HCl
3. Autoclaved at 250°F for 20 minutes

Bufferfields's Phosphate Buffer

Stock solution was prepared by dissolving 34 g of KH_2PO_4 (Merck) in 500 ml of distilled water; adjusted to pH 7.2 with about 175 ml of 1N NaOH and diluted to 1 liter with distilled water. This stock solution was kept under refrigeration at 40°F. The prepared diluent was made by adding 1.25 ml of the stock solution per liter of distilled water.

VITA

Selvestion Jimes is the son of Mr. and Mrs. Raymond Jimes of Bossier City, Louisiana. He was born in Shreveport, Louisiana on July 21, 1937. He graduated from Bossier High School in Bossier City, Louisiana on May 23, 1955. In September of 1955, he entered Northwestern State College of Natchitoches, Louisiana, from which school he graduated with a B.S. degree in chemistry and bacteriology on June 2, 1959. He was commissioned as a 2nd Lieutenant in the Artillery and Guided Missle Corp of the United States Army on that date.

He was employed in the Confederate Memorial Medical Center Laboratory in Shreveport, Louisiana from July 1, 1959 until February 6, 1960, at which time he went on active duty for a six month tour in the United States Army at Fort Sill, Oklahoma.

In 1961, he was married to former Miss Patsy Ann Goldsby, daughter of Mr. and Mrs. J. W. Goldsby of Mansfield, Louisiana.

On January 29, 1962, he received a Master of Science degree from Northwestern State College of Louisiana with a Bacteriology major and a Chemistry minor.

February 12, 1962, a daughter named Janet Adrienne Jimes was born.

From March 1962 to August 1964, he was employed as a chemist and microbiologist for the United States Federal Food and Drug Administration.

In September of 1964, he entered L.S.U. Graduate School in the Department of Food Science and Technology.

On May 31, 1966 he was promoted to the rank of Captain in the United States Medical Service Corp Stand By Reserve.

EXAMINATION AND THESIS REPORT

Candidate: Selvestion Jimes

Major Field: Food Science & Technology

Title of Thesis: *Clostridium Botulinum* Type E in Gulf Coast Shrimp and Shucked Oysters, and Toxin Production as Affected by Irradiation Dosage, Temperature, Storage Time and Mixed Spore Concentrations

Approved:

Robert M. Grodner
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May 9, 1967